

Stimulation of DNA synthesis in rat and mouse liver by various tumor promoters

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In order to investigate whether the stimulation of liver DNA synthesis might be used to detect one class of hepatic tumor promoters, the incorporation of orally administered radio-labelled thymidine into liver DNA was determined in rats and mice 24 h after a single oral gavage of test compounds at various dose levels. Three DNA-binding hepatocarcinogens, aflatoxin B₁, benzidine and carbon tetrachloride, did not stimulate but rather inhibited DNA synthesis (not for CCl₄). Four hepatic tumor promoters, clofibrate, DDT, phenobarbital and thioacetamide, gave rise to a stimulation in a dose-dependent manner. Single oral doses between 0.02 and 0.3 mmol/kg were required to double the level of thymidine incorporation into liver DNA (= doubling dose, DD). Differences between species or sex as observed in long-term carcinogenicity studies were reflected by a different stimulation of liver DNA synthesis. In agreement with the bioassay data, aldrin was positive only in male mice (DD = 0.007 mmol/kg) but not in male rats or female mice. 2,3,7,8-TCDD was positive in male mice (DD = 10⁻⁶ mmol/kg) and in female rats (DD = 2 × 10⁻⁶ mmol/kg) but not in male rats. The assay was also able to distinguish between structural isomers with different carcinogenicities. [alpha]Hexachlorocyclohexane stimulated liver DNA synthesis with a doubling dose of about 0.2 mmol/kg in male rats whereas the [gamma]-isomer was ineffective even at 1 mmol/kg. So far, only one result was inconsistent with carcinogenicity bioassay data. The different carcinogenicity of di(2-ethylhexyl)adipate (negative in rats) and di(2-ethylhexyl)phthalate (positive) was not detectable. Both plasticizers were positive in this short-term system with DD's of 0.7 mmol/kg for DEHA and 0.5 mmol/kg for DEHP. The proposed assay is discussed as an attempt to devise short-term assays for carcinogens not detected by the routine genotoxicity test systems.

Introduction

A number of short-term tests exist for the assessment of the carcinogenic potential of chemicals. Most of these detect only carcinogens which interact with nucleic acids (1), or potentially induce DNA repair synthesis (2) or mutations in bacteria or mammalian cells (3,4). An increasing number of compounds are being found to induce tumors in a long-term bioassay although they generally produce negative results in the above genotoxicity tests.

***Abbreviations:** CLF, clofibrate; PB, phenobarbital; DD, doubling dose; AFB₁, aflatoxin B₁; BZD, benzidine; [alpha]HCH, [alpha]hexachlorocyclohexane; [gamma]HCH, [gamma]hexachlorocyclohexane; ALD, aldrin; DDT, 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; DEHP, di(2-ethylhexyl)phthalate; TAA, thioacetamide; DEHA, di(2-ethylhexyl)adipate; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; [¹⁴C]TdR, [methyl-¹⁴C]thymidine; [³H]TdR, [methyl-³H]thymidine; TdRII, thymidine incorporation index; SF, stimulation factor.

They are often active as tumor promoters in two-stage experiments and exhibit biological activities as hormones (e.g. ethinylestradiol), peroxisome proliferators [e.g. clofibrate (CLF*)] or enzyme inducers [e.g. phenobarbital (PB)]. A correlation of these activities with the carcinogenicity of these compounds might be empirical but it could form the basis for short-term assays. At the present time only the initiation-promotion assay is employed routinely in the liver (5–7). The test compounds are examined for their ability to promote tumor or foci formation after initiation with a known genotoxic agent. In the search for a potentially common biological activity of some tumor promoters, our decision fell on the stimulation of DNA synthesis. Although not a sufficient condition (8,9), cell division seems to be a prerequisite in a number of stages in the process of carcinogenesis. Firstly, the replication of damaged DNA is a necessary event for a successful initiation (10–13). Secondly, the mitotic activity plays an important role during the period of tumor promotion and progression (14).

In this report we investigated the ability of different hepatocarcinogens to stimulate DNA synthesis in the liver of rats and mice. The experimental system elaborated by Schulte-Hermann and co-workers (15) was applied. Radiolabelled thymidine incorporation was measured in the peak phase of the diurnal cycle of liver DNA synthesis, 24 h after administration of the test compounds at various dose levels. The dose which produced a doubling of the control level DNA synthesis was named the doubling dose (DD) and was used for quantitative comparisons. In a first set of experiments, DNA-binding carcinogens were compared with classical hepatic tumor promoters to check whether it is possible to distinguish between the two classes. The system was tested further by using carcinogens with different potencies in different species (rats versus mice) or sex. Non-carcinogenic structural isomers of hepatocarcinogens were also investigated.

Materials and methods

Animals

Rats were from Ivanovas, Kisslegg, FRG (Iva:SIV-50 SD), from the University Hospital Zürich, Switzerland (Osborne-Mendel) or from Charles River, Sulzfeld, FRG [CrI:CD(SD)BR or CDF(F-344)/CrIBR]. Mice were from Charles River, Sulzfeld, FRG (B6C3F₁/CrIBR).

The animals were kept in Marcolone cages, the rats in pairs, the mice in groups of four. The illumination time was from 9 a.m. to 9 p.m. Food pellets (Haltungsdiät Nr. 343, Klingental Mühle AG, Kaiseraugst, Switzerland) in unlimited quantity were available only between 9 p.m. and 2 a.m. For certain control experiments, food availability was extended to 15 h (9 p.m. to 12 a.m.). Drinking water was available at all times. The animals were kept untreated for an acclimatization period of at least 2 weeks, whereafter weight gain was as high as without restricted diet. Treatment groups usually consisted of four animals, in some cases there were only two animals per group (aflatoxin B₁ [AFB₁], benzidine [BZD], CCL₄, CLF, PB, [alpha]hexachlorocyclohexane [[alpha]HCH], [gamma]hexachlorocyclohexane [[gamma]HCH]). The animals' weight was recorded twice a week.

Chemicals

The following test compounds were used: AFB₁ (Senn AG, Dielsdorf, Switzerland), aldrin (ALD) (Riedel De Haen AG, Seelze, FRG), BZD (Fluka AG, Buchs, Switzerland), CCL₄ (Merck, Darmstadt, FRG), CLF, 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), di(2-ethylhexyl)phthalate (DEHP)

and thioacetamide (TAA) (all from Fluka AG, Buchs, Switzerland), di(2-ethyl-hexyl)adipate (DEHA) (Merck, Darmstadt, FRG), [α]HCH and [γ]HCH (Celamerck GmbH Co., Ingelheim/Rhein, FRG), PB (Siegfried AG, Zofingen, Switzerland), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Givaudan AG, Dübendorf, Switzerland).

Application solutions were made up in corn oil (ALD, CLF, CCl₄, DEHA, DEHP, [α]HCH, [γ]HCH, DDT, TCDD), in ethanol (AFB₁, BZD) or in 0.9% aqueous NaCl (PB, TAA).

Radiolabelled thymidine and methanol were from The Radiochemical Centre, Amersham, UK.

Treatments

Test compounds were administered p.o. at 8 a.m. at the dose levels indicated in the tables. For most carcinogens which do not bind to DNA, the highest dose level chosen approximated the TD₅₀, i.e. the daily dose which would induce a tumor in 50% of the animals when applied daily over their lifetime. Intermediate and low doses, if applicable, were one tenth and one hundredth thereof. Groups of control animals received the vehicle only. The highest dose level of [γ]HCH was above the LD₅₀ range. The animals did not show any sign of toxicity, however, under the conditions of this assay and the liver DNA synthesis after 24 h was still at 80% of control.

Twenty-four hours later all animals were given radioactive thymidine p.o. Rats received 2–5 μ Ci/kg [methyl-¹⁴C]thymidine ([¹⁴C]TdR) (54 mCi/mmol) or 6–15 μ Ci/kg [methyl-³H]thymidine ([³H]TdR) (40 Ci/mmol), mice received 10 μ Ci/kg [¹⁴C]TdR (54 mCi/mmol) in 0.9% aqueous NaCl. For certain control experiments the animals were administered 140 μ Ci/kg [¹⁴C]methanol (59 mCi/mmol), or, for autoradiographic investigations, 1 mCi/kg [³H]TdR (5 Ci/mmol). When using ¹⁴C-TdR, one animal of each dose group and one control was placed in a glass metabolism cage, where the carbon dioxide expired was trapped with ethanolamine/methanol 1:4. Aliquots of this solution were counted for ¹⁴C radioactivity 3 h after the TdR administration. Four hours after thymidine administration the animals were killed by an ether overdose.

DNA isolation

The livers were excised and minced and DNA was isolated by an abbreviated standard procedure (16), essentially by phenol extraction and hydroxylapatite adsorption chromatography.

An aliquot of the DNA was counted for radioactivity after addition of 10 ml Insta-Gel (Packard Instruments, Downers Grove, USA) by liquid scintillation counting in a Packard model 460 CD equipped with and calibrated for the determination of ³H/¹⁴C-double-labelled samples. The amount of DNA was assessed on the basis of the u.v. absorbance at 260 nm, taking an extinction value of 20 for a solution of 1 mg/ml.

Liver histology and autoradiography

Fresh liver tissue of two animals in each group treated with DDT, TAA, ALD, TCDD, DEHA and DEHP was fixed in formalin, embedded in paraffin, cut into sections of 5 μ m thickness and stained with hematoxylin and eosin.

For autoradiography, the 5 μ m sections were dipped in photographic emulsion (Ilford K2-Gel, Ilford Ltd, Basildon, Essex, UK). Exposure time at 4°C was 2–3 weeks.

Calculations and statistics

The specific activity of the DNA (expressed in d.p.m./mg) was divided by the dose of thymidine radioactivity administered (expressed in d.p.m./kg) and this value was multiplied by 3.09×10^8 in order to convert the data to the molar units of a thymidine incorporation index. TdRII = (μ mol TdR incorporated per mol diphosphate)/(mmol TdR administered per kg body wt).

The multiplication factor between the mean TdRII of the untreated control group and the treated groups was called the stimulation factor (SF). The *t*-test was used for the assessment of statistical significance (*P*). The dose required for each compound to induce a doubling of the liver DNA synthesis, the DD, has an SF of 2.

The symbols –, + to +++++, indicating approximate carcinogenic potency in the tables, were assigned according to TD₅₀ values (17) [expressed in mmol/kg(days)]: –, no significant increase under the bioassay conditions used; +, $10 > \text{TD}_{50} > 1$; ++, $1 > \text{TD}_{50} > 0.1$; +++, $0.1 > \text{TD}_{50} > 0.01$; +++++, $0.01 > \text{TD}_{50} > 0.0001$; ++++++, $0.0001 > \text{TD}_{50} > 0.00001$. For the carcinogenic potency ranking used in our quantitative evaluation the chemicals are therefore specified as carcinogens rather than as promoters.

Results

DNA-binding carcinogens versus tumor promoters

The first question to answer was whether the proposed system was specific for carcinogens acting without covalent DNA binding.

Table I shows that the three DNA-binding carcinogens AFB₁ (18a), BZD (18a,e) and CCl₄ (18a,c) did not stimulate cell divi-

Table I. Correlation of carcinogenic potency with stimulation of liver DNA synthesis; DNA-binding agents

Compound	Species/sex strain	Carcinogenic potency	Dose (mmol/kg)	Stimulation factor	Doubling dose (mmol/kg)
Aflatoxin B ₁	Rat/male SIV-50 SD	+++++	0.001	0.4 ^{a*}	Inhibition
			0.0001	0.3 ^{a**}	Inhibition
			0.00001	0.2 ^{a**}	Inhibition
Benzidine	Rat/male SIV-50 SD	+++	1.0	0.5 ^{a*}	Inhibition
			0.1	0.3 ^{a**}	Inhibition
			0.01	0.8 ^a	
CCl ₄	Rat/male SIV-50 SD	+	1.0	1.0 ^a	
			0.1	1.2 ^a	
			0.01	1.5 ^a	

The stimulation factor indicates the ratio of the thymidine incorporation in treated animals over the value in the related control group.

*0.1 $\geq P > 0.05$; ** $P < 0.05$ (*t*-test).

^aThe related control group (Table VI).

Table II. Correlation of carcinogenic potency with stimulation of liver DNA synthesis; hepatic tumor promoters which do not bind to DNA

Compound	Species/sex strain	Carcinogenic potency	Dose (mmol/kg)	Stimulation factor	Doubling dose (mmol/kg)
Clofibrate	Rat/male SIV-50 SD	++	1.0	3.4 ^{a**}	0.1
			0.1	2.2 ^{a**}	
			0.01	0.8 ^a	
DDT	Rat/male CD(SD)	++	0.05	2.8 ^{c*}	0.02
			0.005	1.4 ^d	
Phenobarbital	Rat/male SIV-50 SD	++	0.1	2.5 ^{a**}	0.1
			0.01	1.1 ^a	
Thioacetamide	Rat/male CD(SD)	+++	0.27	2.0 ^{a*}	0.3
			0.027	1.6 ^a	

*0.1 $\geq P > 0.05$; ** $P < 0.05$ (*t*-test).

^{a,c,d,g}The related control group (Table VI).

sion. AFB₁ and BZD actually inhibited DNA synthesis. This has been observed frequently with genotoxic agents as a first cellular response. On the other hand, four classical hepatocarcinogens which do not bind to DNA and which are negative in most tests of mutagenicity, CLF (18d), DDT (19), PB (19) and TAA (20), gave rise to a 2.0–3.4-fold stimulation of DNA synthesis at the highest dose level (Table II). The approximate interpolated DD for these agents ranged between 0.02 and 0.3 mmol/kg with DDT being the most potent.

Species and sex differences

ALD induces liver tumors in male mice, but not in female mice or in rats (21b). The assay was able to reproduce this difference. DNA synthesis was stimulated only in male mice (Table III). Here, ALD was a potent stimulator with a doubling dose of 0.007 mmol/kg.

TCDD is a carcinogen for male mice and female rats, but not for male rats (22a). Again, TCDD was negative in male rat liver, but was a very potent stimulator of DNA synthesis in male mice with a DD of 10^{-6} mmol/kg and in female rats with a DD of 2×10^{-6} mmol/kg (Table III). TCDD, measured in mice, was the most potent stimulator of all compounds tested. TCDD had been tested before in rats and was found to be negative for male and female rats (23). The discrepancy could be explained by the

Table III. Correlation of carcinogenic potency with stimulation of liver DNA synthesis; compounds with different carcinogenic potency in different species and sexes

Compound	Species/sex strain	Carcinogenic potency	Dose (mmol/kg)	Stimulation factor	Doubling dose (mmol/kg)
Aldrin	Rat/male OM	—	0.016	1.3 ^b	
	Mouse/male B6C3F ₁	+++	0.011	3.1 ^{f*}	0.007
	Mouse/female B6C3F ₁	—	0.008	1.2 ^j	
TCDD	Rat/male CD(SD)	—	0.00003 0.000003	1.1 ^f 1.5 ^f	
	Rat/female CD(SD)	+++++	0.0000008	1.5 ^{h*}	0.000002
	Mouse/male B6C3F ₁	+++++	0.0000008	1.7 ^{k**}	0.000001

*0.1 ≥ *P* > 0.05; ***P* < 0.05 (*t*-test).^{b,f,h,i,j,k}The related control group (Table VI).**Table IV.** Correlation of carcinogenic potency with stimulation of liver DNA synthesis; structural isomers with different carcinogenicity

Compound	Species/sex strain	Carcinogenic potency	Dose (mmol/kg)	Stimulation factor	Doubling dose (mmol/kg)
[alpha]HCH	Rat/male SIV-50 SD	++	1.0	3.4 ^{a**}	0.2
			0.1	1.7 ^{a**}	
			0.01	1.4 ^a	
[gamma]HCH	Rat/male SIV-50 SD	—	1.0	0.8 ^a	
			0.1	0.9 ^a	
			0.01	1.3 ^a	

*0.1 ≥ *P* > 0.05; ***P* < 0.05 (*t*-test).^aThe related control group (Table VI).

fact that these authors measured DNA synthesis 35–36 h after TCDD administration, whereas we measured after 24 h, which was found to coincide with the peak DNA synthesis in our system. Also, the dose used by these authors was 40 times larger than ours and it is possible that a potential toxicity interfered with the induction of DNA synthesis.

Structural isomers

It was investigated whether the test system was able to reflect the differences in carcinogenicity of structurally related compounds. Table IV summarizes the results obtained with the structural isomers [alpha]- and [gamma]HCH. Whereas [gamma]HCH has no carcinogenic activity in rats (21a), the isomer [alpha]-HCH is carcinogenic (18b,c). Only the carcinogenic [alpha]HCH isomer stimulated DNA synthesis with a DD of 0.2 mmol/kg. The non-carcinogenic isomer [gamma]HCH did not have an effect on DNA synthesis.

The results obtained with a second pair of structurally related compounds are shown in Table V. DEHP is a liver carcinogen in rats (22c), while DEHA has no such effects in rats (22b). Both compounds were able to stimulate DNA synthesis with DD's of 0.5 mmol/kg for DEHP and 0.7 mmol/kg for DEHA. DEHA was the only compound tested so far where liver tumor induction and stimulation of DNA synthesis were not correlated.

Table V. Correlation of carcinogenic potency with stimulation of liver DNA synthesis; structurally related compounds

Compound	Species/sex strain	Carcinogenic potency	Dose (mmol/kg)	Stimulation factor	Doubling dose (mmol/kg)
DEHA	Rat/male F-344	—	3.78	10.5 ^{e**}	0.7
DEHP	Rat/male F-344	+	1.73	7.8 ^{e**}	0.5

*0.1 ≥ *P* > 0.05; ***P* < 0.05 (*t*-test).^eThe related control group (Table VI).

Control experiments

Bioavailability of radiolabelled TdR. All results are based on measurements of the specific activity of liver DNA (d.p.m./mg) arising from the incorporation of methyl radiolabelled thymidine after oral gavage. Exhalation of ¹⁴CO₂ as a consequence of enzymatic thymidine degradation was routinely checked in order to assure that the bioavailability of the radiolabelled DNA precursor was uniform amongst the animals. On an average, 16 ± 4% of the administered radioactivity in rats and 43 ± 5% in mice was recovered as ¹⁴CO₂ within 3 h.

Autoradiography. To prove that an increase in DNA radioactivity was correlated with an increase in dividing hepatocytes, a parallel experiment with an autoradiographic evaluation was conducted with 0.05 mmol/kg DDT. The fraction of cells in S-phase was 0.51%, control liver showed 0.15%. The SF by determination of the specific activity of the DNA and by autoradiographic analysis were therefore on a similar level.

Histology. Literature data show that regenerative DNA synthesis in rat liver after single administration of a necrogenic dose of CCl₄ does not start until 30 h after the administration (24). A separation between adaptive (= promotive?) and regenerative liver DNA synthesis therefore seems possible. Nevertheless, in order to exclude the possibility that an increase in DNA radioactivity was only the result of regenerative processes after cell death induced by the test compounds, histological preparations of the livers were examined for necrotic regions after treatment with DDT, TAA, ALD, TCDD, DEHA and DEHP. An increase in necroses was never found.

Interindividual variability. In all experiments there was a large variability of the TdRII for equally treated animals. The standard deviation was on average about 50% of the mean value. The variability was not treatment-related, being also found in the controls. Table VI summarizes the mean values and standard deviations of the TdRIIs of all control groups. The means range from 2680 to 5540 in rats and from 850 to 1310 in mice. It was therefore necessary to relate the results of each experiment to its own control group. Unsuccessful attempts to reduce the variability will be discussed.

Discussion

It has been known for a long time that many xenobiotics induce liver growth. The topic has been comprehensively reviewed by Schulte-Hermann (25,26). The role of liver growth in the process of carcinogenesis has been studied primarily on a mechanistic and cellular level and relatively little effort has been made to evaluate this response of the liver for toxicological purposes. In our assays, liver weights were not increased above control. This lack of an observable effect is most probably due to the short

Table VI. Incorporation of [14 C]TdR into liver DNA of untreated control groups

Group (index)	Mean value of incorporation index	Absolute standard deviation	n	Relative standard deviation (%)
<i>A. Rats</i>				
a	2680	980	4	37
b	4370	2570	4	59
c	5540	4360	4	79
d	5080	3880	4	76
e	4600	1860	4	40
f	4270	1730	4	41
g	4280	3130	4	73
h	3460	430	4	12
<i>B. Mice</i>				
i	850	580	3	67
j	1130	640	3	56
k	1310	260	4	20

period of time between the administration of the test compound and the isolation of the liver DNA.

We have tried to characterize the liver's response in a quantitative way by comparing the doses required for various compounds to double the control level of DNA synthesis. Our results indicated in most cases not only a qualitative but also a quantitative correlation between the carcinogenic potency and the DNA synthesis stimulating potency (expressed as the DD) of carcinogens which do not act by DNA binding. Compounds with a high carcinogenic potency were active at lower dose levels to stimulate DNA synthesis than compounds with a lower potency. DNA-binding carcinogens did not stimulate DNA synthesis at all. Therefore the stimulation test could represent a useful tool for detecting a number of carcinogens which are missed by the routine genotoxicity test systems.

Furthermore, we checked whether compounds that induce liver tumors only in one rodent species, or only in one sex would stimulate DNA synthesis in the susceptible but not in the resistant case. For the compounds investigated in this study, it was possible to pinpoint mouse- or rat-specific carcinogens and sex-specific carcinogens. A difference in carcinogenicity of structurally-related compounds was reflected by the assay with the HCH isomers but not with the plasticizers DEHP and DEHA. Further work is necessary to elucidate this 'false positive' situation.

Attempts to reduce the interindividual variability

A large interindividual variability sometimes unacceptably reduced the level of significance of an effect. Various means were tested to reduce this drawback: (1) [14 C]methanol was used instead of [14 C]thymidine to label all four nucleotides via the carbon-1 pool; (2) only brothers were taken in a treatment group, to have genetically similar animals; (3) food was available for 15 h instead of 5 h in order to reduce the stress of hunger; (4) 'internal standardization': at day 0 at 8 a.m., the animals were administered [3 H]TdR, to determine the level of DNA synthesis before treatment with the test compound. Twenty-four hours later the animals were given the test compound. After additional 24 h, they received [14 C]TdR, to determine the level of DNA synthesis after the treatment with the test compound. On the basis of the 3 H- and the 14 C-counts in each liver DNA every animal served as its own control. Unfortunately, none of these procedures resulted in a reduction of the variability (data not shown).

Combination with short-term tests for genotoxicity and future developments

The assay proposed here seems to be useful to recognize some of those carcinogens which are notoriously missed by tests for genotoxicity. The concept of the doubling dose (i.e. the single dose of a compound required to double the control level of DNA synthesis) might even allow a quantitative evaluation. Parodi and co-workers have shown that a combination of two short-term tests leads to an improvement of the predictivity if both tests can be evaluated quantitatively and if both tests correlate to some extent with carcinogenic potency. The improvement will be maximal if the two tests recognize different classes of carcinogens, i.e. do not correlate with each other (27). A preliminary analysis of the present data showed that a combination of the DD with a quantitative short-term test for genotoxicity, for instance with a test measuring covalent DNA binding *in vivo* (1) indeed markedly improved the prediction of the potency of a carcinogen on the basis of a combination of two short-term tests (28).

The results show that a single administration of the test compound is sufficient to induce an observable effect and to provide a surprisingly good correlation with carcinogenic potency. It might be expected that a repeated application would reflect the real situation even better. It is also imaginable that this could reduce the interindividual variability, because fluctuations from day to day would be averaged out. On the other hand, the costs for performing the test would be greater than with a single application.

With the compounds investigated so far, only one false positive and no false negative result was found. This is an astonishingly good result. It is probable, however, that false negative results sometimes will also be found, because no short-term test can be expected to reflect all aspects and stages of carcinogenesis by chemicals. It could, for instance, be imagined that a compound leads to an increased formation of peroxides and oxygen radicals able to damage DNA. This indirect genotoxicity would go unobserved in a DNA binding study and possibly also in a test on DNA synthesis, although it was recently reported that hydroperoxides of fatty acids stimulated DNA synthesis in rat colon (29). It will then be necessary to search for the biological activity responsible in that specific situation for the tumors induced, and to try to develop a corresponding short-term test. This would finally allow the replacement of the long-term bioassay for carcinogenicity by a battery of test systems with complementary scope. The mechanistic information available from such a combination of assays would also be of value for extrapolations to man.

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